

# VU Research Portal

## Surgery-induced liver metastasis

Gül, N.

2012

### **document version**

Publisher's PDF, also known as Version of record

[Link to publication in VU Research Portal](#)

### **citation for published version (APA)**

Gül, N. (2012). *Surgery-induced liver metastasis: how it begins and how to end it*. [PhD-Thesis - Research and graduation internal, Vrije Universiteit Amsterdam].

### **General rights**

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal ?

### **Take down policy**

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

### **E-mail address:**

[vuresearchportal.ub@vu.nl](mailto:vuresearchportal.ub@vu.nl)

*Intravital microscopy identifies  
antibody-dependent phagocytosis by  
macrophages as main effector  
mechanism to prevent development  
of liver metastases*

Nuray Güll<sup>1</sup>, Kerstin Siegmund<sup>2</sup>, Marijn Bögels<sup>1,2</sup>, Rens Braster<sup>1</sup>, Gestur Vidarsson<sup>4</sup>, Paul Kubes<sup>3</sup>, Marjolein van Egmond<sup>1,2</sup>

<sup>1</sup>Department of Molecular Cell Biology and Immunology, VU University Medical Center, Amsterdam, The Netherlands,

<sup>2</sup>Department of Surgery, VU University Medical Center, Amsterdam, The Netherlands,

<sup>3</sup>Immunology Research Group, University of Calgary, Canada

<sup>4</sup>Department of Experimental Immunohematology, Sanquin Research, and Landsteiner Laboratory, Academic Medical Center, University of Amsterdam, Plesmanlaan 125, Amsterdam 1066 CX, The Netherlands.

Submitted

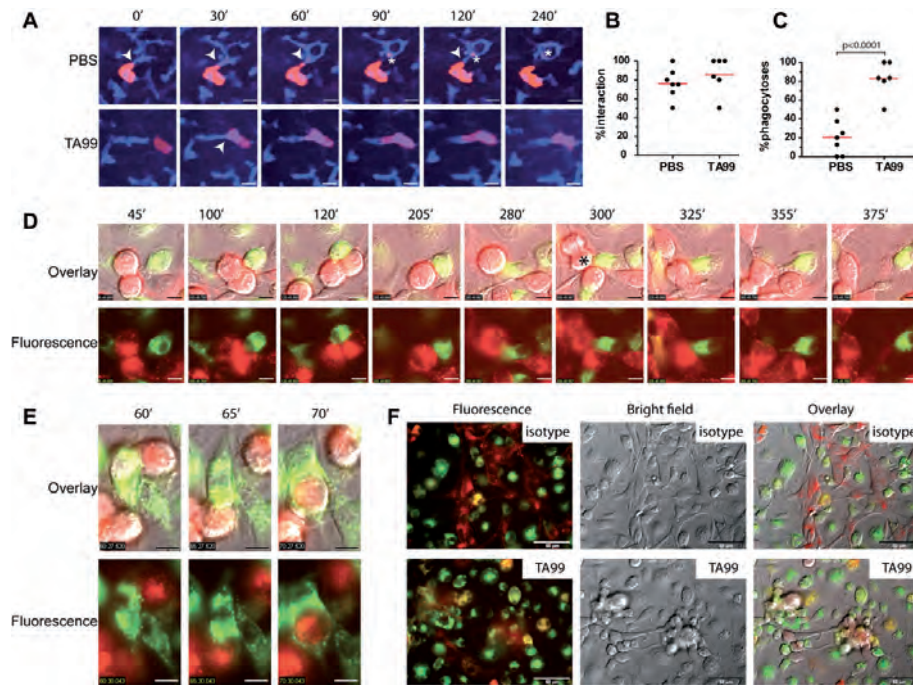
## Introduction

Monoclonal antibodies (mAbs) directed against tumor antigens are currently used as promising adjuvant therapies in addition to radio- and/ or chemotherapy.<sup>1</sup> Although clinical successes have been demonstrated, it is not completely understood how mAbs mediate tumor elimination. We previously demonstrated that mAb therapy is particularly successful in preventing surgery-induced liver metastases development in animal models.<sup>2,3</sup> In the current study we used real-time intra-vital microscopy to elucidate the mechanisms that are involved in removal of tumor cells. We demonstrate that tumor cells are rapidly recognized and arrested by liver macrophages (Kupffer cells). Kupffer cells sampled tumor cells in the absence of mAb, but this did not lead to elimination. By contrast, mAb treatment resulted in fast phagocytosis of tumor cells by Kupffer cells, followed by degradation. As such, we propose that macrophages play a much more prominent role in mAb-mediated eradication of tumor cells in patients than is currently appreciated. With the use of intra-vital microscopy we show for the first time directly that antibody-dependent phagocytosis by macrophages is a prominent mechanism to induce therapeutic efficacy of mAbs. These findings may help to develop optimized mAb therapeutical strategies of many cancer patients, by aiming to enhancing macrophage recruitment and activity.

Therapeutic monoclonal antibodies (mAb), which can be designed to specifically interact with tumor-associated antigens, represent a promising novel category of drugs for targeting malignancies in addition to chemo- or radiotherapy.<sup>4</sup> The anti-CD20 mAb rituximab was one of the first that was approved for clinical use to treat B cell malignancies,<sup>5</sup> and its unprecedented success prompted the development of a multitude of new anti-tumor mAb. The therapeutic mode of action of mAb is still incompletely understood and heavily debated, in spite of an overwhelming number of *in vitro*, *in vivo* and patient studies.<sup>6</sup> Several direct and indirect mechanisms of mAb therapy have been proposed. Direct mechanisms include the induction of apoptosis, inhibition of proliferation or sensitization of tumor cells for chemotherapy.<sup>4,5</sup> Furthermore, most mAb that are used in the clinic are of the IgG1 subclass, which activates the complement cascade through the classical pathway, leading to complement-dependent lysis. The Fc region of IgG additionally interacts with IgG Fc receptors (FcγR) that are expressed on immune cells. FcγR-mediated mechanisms proved essential for therapeutic efficacy *in vivo*, since mAb immunotherapy was ineffective in FcγR deficient mice.<sup>7</sup> A strong association between certain FcR alleles and success of mAb therapy in patients was shown as well, as patients with the FcγRIIIa-158V allotype had a better clinical response compared to FcγRIIIa-158F allotype.<sup>5</sup> This effect has mainly been attributed to FcγRIII- expressing natural killer (NK) cells, which ability to lyse mAb-opsonized target cells is referred to as antibody-dependent cellular cytotoxicity (ADCC).<sup>4</sup> Recently, a role for macrophages was proposed as depletion with

clodronate liposomes abrogated therapeutic efficacy of anti-murine CD20 mAb in a murine lymphoma model.<sup>8</sup>

Notwithstanding the initial success of treating haematological malignancies with mAbs, therapeutic accomplishments in targeting solid tumors remain somewhat disappointing.<sup>1</sup> This is partly explained by the lack of efficacy when direct effects of mAb are inhibited. For instance, the anti-HER-2 mAb trastuzumab is only effective when HER-2 is over-expressed, which is the case in ~20-25% of breast cancer patients, whereas mutations in the KRAS signaling pathway seriously hamper therapeutic success when colorectal patients are treated with anti-EGFR mAb.<sup>9</sup> Understanding the precise mode of action of mAb therapy will allow the design of optimal mAb therapeutical strategies to improve efficacy in these patients.



**Figure 1:** mAb induce ADPh of tumor cells by macrophages. (a) Interaction between Kupffer cells and B16F10 tumor cells in livers of mice that were either treated with isotype (upper panels) or TA99 (lower panels) mAb. Arrowheads indicate contact site between Kupffer cell and tumor cell, whereas the asterisks show uptake of small tumor cell particles. Scale bar 25 mm. (b) quantification of interactions between Kupffer cells and tumor cells. (c) quantification of phagocytosis of B16F10 tumor cells by Kupffer cells. (d-f) *in vitro* live cell microscopy of DiO-labeled macrophages (green fluorescence) and Dil-labeled B16F10 cells (red fluorescence) in the presence of (d) isotype or (e) TA99 mAb. Time points are indicated. (d+e) Upper panels: overlay of bright field and fluorescence. Lower panels: fluorescence only. Asterisk in d indicates tumor cell division (f) live cell microscopy images after 24 hours. Upper panels; incubation with isotype control, lower panels; incubation with TA99 mAb. Left panels; fluorescence, middle panels, bright field, right panels, overlay. Data are representative of five independent experiments.

We previously identified a novel mAb-based therapeutic strategy that may benefit a significant population of cancer patients.<sup>3</sup> Approximately one million patients worldwide are diagnosed with colorectal cancer annually, and ~500,000 patients die from this disease each year.<sup>10</sup> Resection of the primary tumor is the cornerstone of the treatment of colorectal cancer. However, disseminated circulating tumor cells can be detected in up to 70% of patients at the time of surgery.<sup>11, 12</sup> The presence of circulating tumor cells has furthermore been proposed as an independent prognostic factor for survival of patients with colorectal cancer.<sup>11, 13</sup>

We recently demonstrated that surgery paradoxically enhances the risk of liver metastases development, as it induces damage to the liver vasculature, which promotes adherence of circulating tumor cells.<sup>14, 15</sup> Importantly, anti-tumor mAb therapy prevented liver metastases outgrowth in mice and rats, which was depended on the presence of either FcγRI or FcγRIV.<sup>2, 3</sup> This supported the involvement of the tissue macrophages of the liver (Kupffer cells), because both receptors are only expressed by the myeloid mononuclear network in mice.

Kupffer cells reside in the liver sinusoids and clear the hepatic blood circulation from bacteria, old blood cells or foreign substances.<sup>16, 17</sup> As such, they are the first line of defense against tumor cells entering the liver. In a previous study, we observed that ~80% of tumor cells were in contact with Kupffer cells in the absence of tumor-specific mAb, which was increased to approximately 90% after mAb treatment.<sup>3</sup> Surprisingly, this modest difference completely prevented liver metastases development. To study the exact mechanisms of mAb therapy, we used real-time intravital microscopy of the liver of mice and *in vitro* live cell imaging and Imagestream technology to visualize the mode of action in more detail.

In the current study the B16F10 melanoma cell line was used as model system, since this is the only syngeneic solid tumor model for which an anti-tumor mAb is available (TA99; anti-gp75). Mice were either treated with an isotype control or with TA99. Real-time imaging in the livers of isotype control treated mice showed that Kupffer cells were able to sample small parts of tumor cells, which however did not result in elimination of tumor cells (Figure 1a, upper panels). Still, these results explained why co-localization between Kupffer cells and tumor cells was observed in previous immunohistochemistry experiments.<sup>3</sup> In contrast, tumor cells were rapidly recognized and phagocytosed in the livers of TA99 treated mice, (Figure 1a, lower panels). Thus, although no difference was observed between the number of tumor cells that was in contact with Kupffer cells after either isotype or TA99 mAb therapy (Figure 1b), the number of tumor cells that had been phagocytosed was significantly increased after TA99 treatment (Figure 1c). Similar results were observed in *in vitro* live cell experiments. Even though cell-cell contact was observed for prolonged periods of time in the absence of tumor-specific mAb, macrophages were unable to prevent tumor growth (Figure 1d and supplementary movies). Addition of TA99 however resulted in rapid phagocytosis and elimination of tumor cells (Figure 1e and f).

Recently, it was suggested that macrophages eliminate tumor cells via ADCC, as synapse formation between tumor cells and macrophages was observed in peritoneal lavages of mAb treated mice.<sup>18</sup> As uptake of tumor cells by macrophages requires a close interaction, synapse formation is likely involved. However, we now show that the main mechanism by which liver macrophages eliminate tumour cells is through antibody-dependent phagocytosis (ADPh). In addition, both murine bone marrow derived and human monocyte derived macrophages killed a wide variety of human tumor cells (colon carcinoma, vulvar carcinoma, B cell lymphoma) through ADPh in live cell imaging experiments (data not shown).

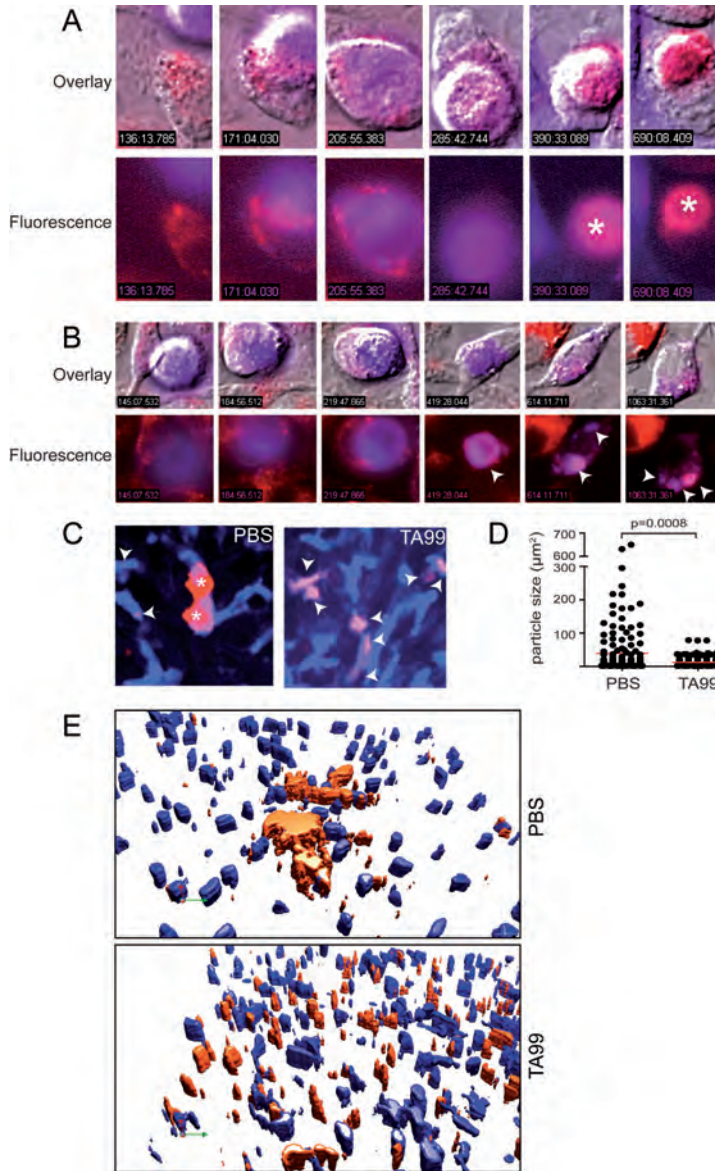
Uptake of tumor cells was associated with phagosome- lysosome fusion, which led to rapid acidification, but slow degradation of tumor cells (Figure 2a and b). This was comparable to intravital experiments. When livers of mice were studied 24 hours after injection of B16F10 cells and TA99, tumor cells had been degraded into smaller particles, but tumor material was still present (Figure 2c; right panel, 2d, 2e; lower panels). Some small tumor particles were observed in Kupffer cells of isotype treated mice, but most tumor cells were present in larger clusters, indicative of tumor cell outgrowth (Figure 2c; left panel, 2d, 2e; upper panels and supplementary movie).

Depletion of Kupffer cells with clodronate liposomes<sup>19</sup> abrogated therapeutic effects of TA99. Large tumor cell clusters were observed in either PBS, isotype control or TA99 mAb treated mice 24 hours after injection of tumor cells in Kupffer cell- depleted mice (Figure 3a). There was no difference in size of tumor cells particles between PBS, isotype control or TA99 treated mice (Figure 3a, right panel). Moreover, whereas treatment with TA99 almost completely prevented outgrowth of liver metastases in control mice, no therapeutic protection was observed in Kupffer cell-depleted mice (Figure 3b).

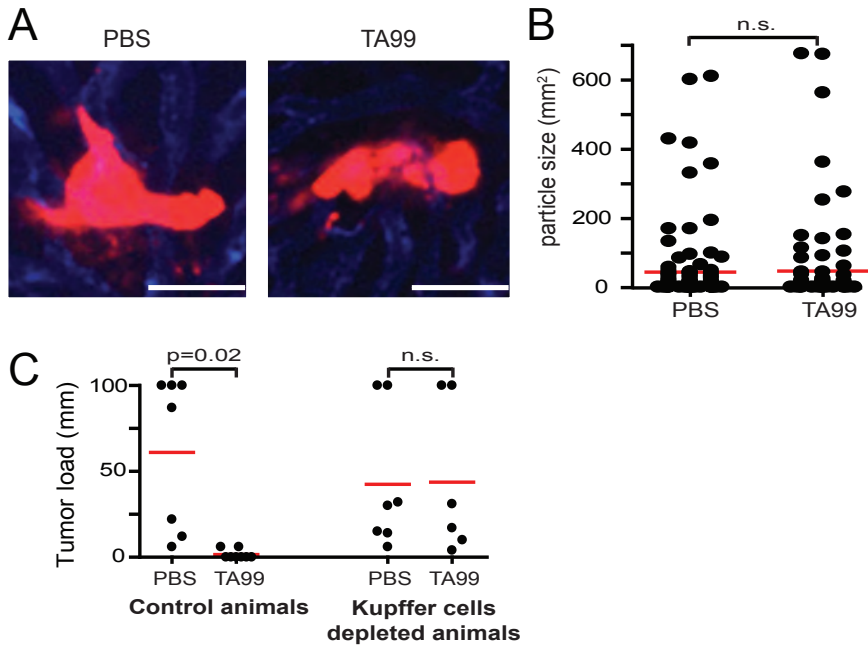
Thus, Kupffer cells are essential in mediating tumor cell elimination after mAb immunotherapy. Until now, modes of action of mAbs have been proposed based on *in vitro* results or experiments with mice that were either deficient for specific components of the immune system, or in which immune components or cells had been depleted. With the use of intravital microscopy we now demonstrate for the first time directly that ADPh by macrophages is crucial for therapeutic efficacy of mAbs. As such, macrophages may play a much more prominent role in mAb-mediated eradication of tumor cells in patients than is currently appreciated. Because human macrophages express FcγRIII, we hypothesize that the difference in therapeutic efficacy of mAbs in patients with distinct Fc receptor allotype may be (partly) attributed to decreased/increased ADPh by macrophages in addition to NK cell- mediated ADCC,<sup>20</sup> particularly because macrophages proved extremely efficient in phagocytosis of B cell lines in the presence of anti-CD20 mAb (data not shown).

Thus, our findings may significantly contribute to the design of optimized mAb





**Figure 2(left page):** Fast uptake, but slow degradation of tumour cells by macrophages. (a+b) Uptake of DiB-labeled (blue fluorescence) B16F10 tumour cell by (a) macrophage labeled with lysotracker (red fluorescence) or (b) Dil- labeled macrophage (red fluorescence) in the presence of TA99mAb. Asterisks in (a) indicate lysosome. Arrow heads in (b) show degraded particles of tumor cell within macrophage. Time points are indicated. Upper panels: overlay of bright field and fluorescence. Lower panels: fluorescence only. (c) Kupfer cells (blue) and B16F10 tumor cells (red) 24 hours after injection of tumor cells in PBS (left panel) or TA99 (right panel) treated mice. Arrow heads in show tumor cell particles within macrophages. Asterisks indicate whole tumor cells. (d) Quantification of size of tumor tumor cell particles in livers of PBS or TA99 treated mice (e) 3D reconstruction of Kupfer cells (green) and B16F10 tumor cells (orange) in PBS (upper panels) or TA99 (lower panels) treated mice. Colors are artificial. Data are representative of three independent experiments.



**Figure 3:** Kupffer cells are the main effector cells in mAb-mediated elimination of tumor cells. (a) B16F10 tumor cells (red) after treatment with either PBS (left panel) or TA99 (right panel) in livers of mice in which Kupffer cells had been depleted. Scale bar 25  $\mu$ m (b) Quantification of size of tumor cells particles after PBS or TA99 treatment in Kupffer cell-depleted mice. (c) Liver metastases development in control or Kupffer cells depleted mice that were treated with PBS or TA99. Data are representative of three independent experiments.

therapeutic strategies of many cancer patients, by aiming to promote recruitment of macrophages as effector cells. In particular, we propose that patients undergoing resection for primary colorectal cancer may greatly benefit from peri-operative mAb immunotherapy, as this will lead to elimination of any remaining circulating tumor cells by Kupffer cells in the liver.

## Material and methods

### Animal models

Wild type C57BL/6 mice were purchased from Charles River. All mice had access to food and water *ad libitum* and were housed in specific pathogen-free, double-barrier units at the University of Calgary in Calgary or VU University Medical Center



in Amsterdam. Protocols were in accordance with guidelines of University of Calgary Animal Care Committee and the Canadian Council on the Use of Laboratory Animals or the Committee for Animal Research of the VU University Medical Center. Mice were used between 8 and 12 weeks of age.

### Cell culture

*The mouse melanoma cell line B16F10*, which expresses gp75, was obtained from the American Type Culture Collection. Cells were cultured in RPMI 1640 medium (Invitrogen, Breda, the Netherlands) supplemented with 10% heat-inactivated FCS and antibiotics, hereafter referred to as complete growth medium. Single cell suspensions were prepared by enzymatic detachment using trypsin-EDTA solution (Gibco, Irvine, UK). Viability was assessed by trypan blue exclusion, which always exceeded 95%. For *in vivo* experiments, B16F10 cells ( $5 \times 10^6$  cells/ml) were labeled fluorescently by incubating cells in complete growth medium containing 50  $\mu\text{g}/\text{ml}$  1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate (Dil, Sigma-Aldrich, St. Louis, MO) for 30 minutes at 37°C and subsequently washed with Hanks' balanced salt solution containing (HBSS; Gibco) according to the manufacturer's instructions.

*Murine bone marrow macrophages.* Bone marrow from wild type C57BL/6 mice was harvested from freshly isolated femur, tibia and humerus. After removal of connective tissues and muscles, bone marrow was flushed and single cell suspensions were made by passing bone marrow through a sterile 70  $\mu\text{m}$  filter (BD Falcon, Bedford, MA). Macrophages were differentiated by incubating bone marrow cells for 7 days with complete DMEM, supplemented with 15 % L929 conditioned medium (containing macrophage-colony stimulating factor). Macrophages were harvested after a 15 minute incubation with trypsin-EDTA and subsequent scraping using a cell scraper. Macrophages were seeded in 24 well plates ( $4 \times 10^5$ / well) for *in vitro* cytotoxicity assays (Imagestream) or in 8 well ibiTreat  $\mu$ -Slides (IBIDI, Munich, Germany) for live cell imaging ( $2 \times 10^5$ / well).

### In vivo experiments

**Intravital microscopy** Animals were anesthetized by an i.p. injection of a mixture of 10 mg/kg xylazine (Bayer, Toronto, Ontario, Canada) and 200 mg/kg ketamine hydrochloride (Bimeda-MTC, Cambridge, Ontario, Canada) prior to surgery. To maintain anesthesia or to inject antibody intravenously, tail vein of mice were cannulated and anesthetics were injected every other 30 minutes. Alexa-647 labeled F4/80 (eBioscience, San Diego, CA) antibody was injected i.v. to stain Kupffer cells. A small incision was made in the left flank to reveal the spleen. Fluorescently labeled B16F10 cells ( $2 \times 10^5$  cells in 100  $\mu\text{l}$ ) were injected intra-splenically at a constant rate to allow flow of tumor cells toward the liver. After one minute, the spleen was removed to prevent bleeding. A midline and a left subcostal incision were made in order to exteriorize the liver and spleen. The hepatic ligaments were dissected and the intestine was covered with a moist gauze. Animals were placed in a left supine

position and the left liver lobe was positioned onto a Plexiglas microscopic stage. The liver surface was then covered with Saran Wrap to hold the organ in position. Mice were injected i.p. on days 0 with 200 µg anti-gp75 TA99 mAb, or as a control with saline (250 µl). In additional experiments, Kupffer cells were depleted by i.v. injection of clodronate (Roche Diagnostics, Mannheim, Germany) encapsulated in liposomes or saline 3 days prior to tumor cells injection. Alternatively, to visualize tumor cells 24 hours after injection, fluorescently labeled tumor cells were injected in the spleen, after which splenectomy was performed and the incision sutured. Mice were to recover for 24 hours after which animals were prepared for intravital microscopy of the liver as described above.

**Liver metastases development** Effect of TA99 on tumor development in control and KCs depleted mice was studied. KCs were depleted as described and all animals were injected i.p. on days 0, 2, and 4, with 200 µg TA99 mAb, or as a control with saline (250 µl). Tumor cells were injected intrasplenically and splenectomy was performed as described here above. After 21 days, mice were sacrificed, and the number of liver metastases was scored in each mouse.

### Quantification

Each contact between KCs and tumor cells was assigned as interaction between these two cells types and phagocytosis of tumor cells by KCs in the livers was determined as co-localization of complete red tumor cells within blue stained KCs. Differences between treatments were quantified in more than five independent experiments with AnalySIS software (Soft Imaging System GmbH).

### In vitro cytotoxicity assays

Real time cytotoxicity assays were performed with a Olympus CellR real-time live-imaging station (type IX81, UPLFLN40xO/1.3 lens, Münster, Germany). Macrophages were either labeled with DiO (green fluorescence), DiI (red fluorescence) or Lyso-ID Red. DiI, DiO, or DiB (blue fluorescence)-labeled B16F10 variant were added in an E:T ratio 15:1 with TA99 or MG-4 (irrelevant isotype control) antibodies. Pictures were taken every 5 minutes with an Olympus ColorView II camera for 3-6 h, followed by a 15 minutes interval for 18-21 hours. Additionally, random pictures were taken after 24 h.

### Statistical analysis

For comparisons between two groups student T-tests were used. Comparisons between multiple groups (>2) were performed with ANOVA. Statistical significance was accepted at  $p < 0.05$ . Results are presented as mean  $\pm$  SEM.

## Reference list

1. Cunningham,D. *et al.* Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. *N. Engl. J. Med.* **351**, 337-345 (2004).
2. Otten,M.A. *et al.* Experimental antibody therapy of liver metastases reveals functional redundancy between Fc gammaRI and Fc gammaRIV. *J. Immunol.* **181**, 6829-6836 (2008).
3. van der Bij,G.J. *et al.* Experimentally induced liver metastases from colorectal cancer can be prevented by mononuclear phagocyte-mediated monoclonal antibody therapy. *J. Hepatol.* **53**, 677-685 (2010).
4. Clynes,R. Antitumor antibodies in the treatment of cancer: Fc receptors link opsonic antibody with cellular immunity. *Hematol. Oncol. Clin. North Am.* **20**, 585-612 (2006).
5. Amoroso,A. *et al.* Understanding rituximab function and resistance: implications for tailored therapy. *Front Biosci.* **16**, 770-782 (2011).
6. Yamada,T. Therapeutic monoclonal antibodies. *Keio J. Med.* **60**, 37-46 (2011).
7. Clynes,R.A., Towers,T.L., Presta,L.G., & Ravetch,J.V. Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets. *Nat. Med.* **6**, 443-446 (2000).
8. Minard-Colin,V. *et al.* Lymphoma depletion during CD20 immunotherapy in mice is mediated by macrophage FcgammaRI, FcgammaRIII, and FcgammaRIV. *Blood* **112**, 1205-1213 (2008).
9. Benvenuti,S. *et al.* Oncogenic activation of the RAS/RAF signaling pathway impairs the response of metastatic colorectal cancers to anti-epidermal growth factor receptor antibody therapies. *Cancer Res.* **67**, 2643-2648 (2007).
10. Jemal,A. *et al.* Global cancer statistics. *CA Cancer J. Clin.* **61**, 69-90 (2011).
11. Sastre,J. *et al.* Circulating tumor cells in colorectal cancer: correlation with clinical and pathological variables. *Ann. Oncol.* **19**, 935-938 (2008).
12. Wind,J. *et al.* Circulating tumour cells during laparoscopic and open surgery for primary colonic cancer in portal and peripheral blood. *Eur. J. Surg. Oncol.* **35**, 942-950 (2009).
13. Rahbari,N.N. *et al.* Meta-analysis shows that detection of circulating tumor cells indicates poor prognosis in patients with colorectal cancer. *Gastroenterology* **138**, 1714-1726 (2010).
14. Gul,N. *et al.* Surgery-induced reactive oxygen species enhance colon carcinoma cell binding by disrupting the liver endothelial cell lining. *Gut*(2011).
15. van der Bij,G.J. *et al.* Blocking alpha2 integrins on rat CC531s colon carcinoma cells prevents operation-induced augmentation of liver metastases outgrowth. *Hepatology* **47**, 532-543 (2008).
16. Dancygier,H. *Microscopic Anatomy* 2010).
17. Paschos,K.A., Majeed,A.W., & Bird,N.C. Role of Kupffer cells in the outgrowth of colorectal cancer liver metastases. *Hepatol. Res.* **40**, 83-94 (2010).
18. Hubert,P. *et al.* Antibody-dependent cell cytotoxicity synapses form in mice during tumor-specific antibody immunotherapy. *Cancer Res.*(2011).
19. van Rooijen ,N. & Sanders,A. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. *J. Immunol. Methods* **174**, 83-93 (1994).
20. Persky,D.O. *et al.* Fc gamma receptor 3a genotype predicts overall survival in follicular lymphoma patients treated on SWOG trials with combined monoclonal antibody plus chemotherapy but not chemotherapy alone. *Haematologica*(2012).